

Neutrophils Alleviate Fibrosis in the CCl₄-Induced Mouse Chronic Liver Injury Model

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Tribbles pseudokinase 1 (*Trib1*) is a negative regulator of CCAAT/enhancer binding protein α (C/EBP α) and is known to induce granulopoiesis while suppressing monocyte differentiation. Loss of *Trib1* was previously shown to increase the neutrophil population in the spleen but lead to M2-like macrophage reduction. Because M2 macrophages are anti-inflammatory and promote tissue repair by producing fibrogenic factors, we investigated liver fibrosis in *Trib1*-deficient mice. Interestingly, loss of *Trib1* suppressed fibrosis in the CCl₄-induced chronic liver injury model. *Trib1* knockout increased neutrophils but had a minimal effect on the macrophage population in the liver. Hepatic expressions of neutrophil matrix metalloproteinases (*Mmp8* and *Mmp9*) were increased, but the production of fibrogenic factors, including transforming growth factor β 1, was not affected by loss of *Trib1*. These results suggest that neutrophils are responsible for the suppression of fibrosis in *Trib1*-deficient liver. Consistently, transplantation of *Trib1*-deficient bone marrow cells into wild-type mice alleviated CCl₄-induced fibrosis. Furthermore, expression of chemokine (C-X-C motif) ligand 1 (*Cxcl1*) by adeno-associated viral vector in the normal liver recruited neutrophils and suppressed CCl₄-induced fibrosis; infusion of wild-type neutrophils in CCl₄-treated mice also ameliorated fibrosis. Using recombinant adeno-associated virus-mediated expression of *Mmp8* and *Mmp9* alleviated liver fibrosis. Finally, neutrophil depletion by infusion of Ly6G antibody significantly enhanced CCl₄-induced fibrosis. **Conclusion:** While neutrophils are well known to exacerbate acute liver injury, our results demonstrate a beneficial role of neutrophils in chronic liver injury by promoting fibrolysis. (*Hepatology Communications* 2018;2:703-717)

Fibrosis is an essential process for tissue repair that encapsulates the damaged area. In the liver, chronic inflammation induced by a variety of causes, such as hepatitis virus, alcohol, drugs, and immune and metabolic disorders, accompanies fibrosis that often advances to cirrhosis.⁽¹⁾ Damaged hepatocytes release factors that activate hepatic stellate

cells (HSCs) directly and indirectly by nonparenchymal cells. Upon activation, HSCs become myofibroblasts and produce extracellular matrix (ECM). Progression of fibrosis is regulated by a balance between production and degradation of ECM. Transforming growth factor β (TGF- β) produced by nonparenchymal cells in damaged liver plays a pivotal role

Abbreviations: α -SMA, α -smooth muscle actin; ALT, alanine aminotransferase; C/EBP α , CCAAT-enhancer-binding protein α ; CD, clusters of differentiation; cDNA, complementary DNA; Cxcl1, chemokine (C-X-C motif) ligand 1; Cyp2e1, cytochrome P450 2E1; ECM, extracellular matrix; FACS, fluorescence-activated cell sorting; HSC, hepatic stellate cell; KO, knockout; MMP, matrix metalloproteinase; MSC, mesenchymal stem cell; qRT-PCR, quantitative reverse-transcription polyacrylamide chain reaction; rAAV, recombinant adeno-associated virus; TGF- β , transforming growth factor β ; *Trib1*, Tribbles pseudokinase 1.

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in fibrogenesis by stimulating production of ECM from HSCs.⁽²⁾

Macrophages also play a central role for pathogenesis of chronic liver injury by phagocytosis of apoptotic liver cells, promoting inflammation, and producing matrix metalloproteinases (MMPs) that are responsible for the degradation of ECM.⁽³⁾ Hepatic macrophages are a heterogeneous cell population that includes the liver resident macrophages, Kupffer cells, and bone marrow-derived macrophages.⁽⁴⁾ As they interact with a variety of cell types, they can be profibrotic as well as antifibrotic.⁽⁵⁾ While classification of macrophages has been a subject of debate, at least two macrophage subtypes have been recognized. M1 macrophages are proinflammatory, whereas M2 macrophages are anti-inflammatory and promote tissue repair by producing fibrogenic factors.⁽⁵⁾ There are also many reports showing a scar-resolving macrophage population that does not fit the M1 or M2 phenotype.⁽⁶⁾

Neutrophils are the most abundant type of granulocytes and are derived from the bone marrow progenitor cells that also give rise to monocytes. Neutrophils are a type of phagocyte and play a major role in defense against bacterial and fungal infection. They are rapidly recruited to the site of injury and are involved in pathogenesis by enhancing inflammation.⁽⁷⁾ In the liver, neutrophils are well known to exacerbate acute liver injury, such as ischemia/reperfusion and alcoholic injury.⁽⁸⁾ However, the role of neutrophils in chronic liver injury accompanying fibrosis remains obscure.

Tribbles pseudokinase 1 (*Trib1*) is one of the three members of the Tribbles family that is characterized by having pseudokinase domains that function as a scaffold.⁽⁹⁾ *Trib1* plays a role in the degradation of CCAAT/enhancer binding protein α (C/EBP α), a crucial transcription factor for myeloid cell development, by recruiting it to E3 ubiquitin ligase. Liver-specific knockout (KO) of *Trib1* increases the hepatic expression of C/EBP α .⁽¹⁰⁾ Loss of C/EBP α expression inhibits the formation of granulocytes and leads to a concomitant increase in self-renewal of hematopoietic

stem cells. Expression of C/EBP α initiates with the commitment of multipotential precursors to the myeloid lineage. C/EBP α is specifically up-regulated during granulocytic differentiation and is rapidly down-regulated during the alternative monocytic pathway. Conditional expression of C/EBP α alone in stably transfected bipotential myeloid cells triggers neutrophilic differentiation.⁽¹¹⁾ Thus, *Trib1* deficiency may exhibit a phenotype similar to overexpression of C/EBP α . In fact, Satoh et al. showed that *Trib1* KO increased the neutrophil population in the spleen whereas M2-like macrophages and eosinophils were reduced.⁽¹²⁾ As M2 macrophages are anti-inflammatory and promote tissue repair by producing fibrogenic factors, we were interested in evaluating liver fibrosis in the *Trib1*-deficient mouse.

CCl₄-induced liver injury has been extensively used as a model of acute and chronic liver injury.⁽¹³⁾ A single injection of CCl₄ induces centrilobular necrosis by a reactive free radical metabolite, which is generated by cytochrome P450 2E1 (Cyp2e1) in centrilobular hepatocytes, and causes transient activation of HSCs as a wound healing response. This is followed by rapid repair of the necrotic area by proliferation of the remaining parenchymal hepatocytes in a few days. However, repeated administration of CCl₄ results in continuous activation of HSCs, leading to fibrosis. In this study, we investigated the role of *Trib1* in fibrosis by using this model. Although the expression level of fibrogenic cytokine TGF- β 1 was comparable between wild-type and *Trib1* KO liver, the fibrotic area was significantly decreased in *Trib1* KO liver. Extensive quantitative reverse-transcription polyacrylamide chain reaction (qRT-PCR) analysis revealed marked increases of the neutrophil MMPs *Mmp8* and *Mmp9* in *Trib1* KO liver. Moreover, transplantation of *Trib1* KO bone marrow cells suppressed liver fibrosis of wild-type liver. Interestingly, recombinant adeno-associated virus (rAAV)-mediated expression of chemokine (C-X-C motif) ligand 1 (*Cxcl1*), a neutrophil

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chemoattractant, also suppressed liver fibrosis. Consistently, adoptive transfer of wild-type neutrophils and rAAV-mediated expression of *Mmp8* and *Mmp9* in CCl₄-induced fibrotic mice dramatically alleviated liver fibrosis. Immunodepletion of neutrophils significantly increased CCl₄-induced liver fibrosis. These results reveal an antifibrotic role for neutrophils in chronic liver injury.

Materials and Methods

MICE

C57BL/6 mice were obtained from CLEA Japan (Tokyo, Japan). *Trib1*-deficient mice were provided by Dr. Akira Shizuo (Laboratory of Host Defense, World Premier International Immunology Frontier Research Center, Osaka University, Japan). Wild-type littermates were used as controls. CD45.1 congenic C57BL/6 mice (RBRC 00144) were provided by the RIKEN BioResource Center through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science, and Technology, Japan. All mice were fed a standard chow diet, maintained on a 12-hour light/dark cycle with free access to food and water unless otherwise indicated, and killed at 2:00 PM in the nonfasted state.

For bone marrow transplantation studies, pregnant Ly5.1 mice were injected subcutaneously with busulfan (15 mg/kg) at days 17 and 18 of gestation, a procedure that ablates hematopoietic stem cells in the neonates. The conditioned neonatal mice were injected intraperitoneally with 1×10^6 unmanipulated bone marrow cells obtained from femurs and tibias of *Trib1*-deficient mice and littermate wild-type mice within 24 hours of birth. All animal experiments were performed in accordance with our institutional guidelines.

INDUCTION OF ACUTE LIVER INJURY AND LIVER FIBROSIS

Acute liver injury was induced by a single intraperitoneal injection of CCl₄. CCl₄ (Wako Pure Chemical, Osaka, Japan) was diluted in corn oil (Wako) to 20% and injected into mice at a dose of 1 mL/kg. Liver fibrosis was induced by repeated injection of CCl₄ 2 times per week for 4 weeks unless otherwise indicated. Livers were harvested 3 days after the final CCl₄ injection.

RECOMBINANT AAV VECTORS

rAAV vectors were produced in human embryonic kidney 293 cells as described.⁽¹⁴⁾ To create the rAAV expression vectors, the green fluorescent protein transgene was removed from plasmid liver-specific promoter 1(pLSP1)-enhanced green fluorescent protein (courtesy of Ian Alexander, Children's Medical Research Institute, Westmead, NSW, Australia) to obtain the rAAV vector backbone. Genes of interest (*Cxcl1*, Cre recombinase [*Cre*], *Mmp8*, *Mmp9*) were amplified by qPCR and cloned into the pLSP1 vector (primer sequences described below). All vectors were pseudoserotyped with adeno-associated virus serotype 8 by calcium phosphate transfection with p5E18-VD2/8 (courtesy of James M Wilson, University of Pennsylvania, Philadelphia, PA) and pXX6-80 (courtesy of Jude Samulski, University of North Carolina, Chapel Hill, NC). The vectors generated were named as rAAV.*Cxcl1*, rAAV.*Cre*, rAAV.*Mmp8*, and rAAV.*Mmp9*, according to the encoded transgene.

INDUCED EXPRESSION OF *Cxcl1*, *Mmp8*, AND *Mmp9* IN HEPATOCYTES

For hepatocyte-specific gene delivery, mice were injected intraperitoneally with rAAV vectors encoding *Cxcl1*, *Mmp8*, or *Mmp9* complementary DNA (cDNA). The primer pairs used for the expression vector are listed in Table 1.

HEMATOXYLIN AND EOSIN, SIRIUS RED, AND IMMUNOSTAINING

Liver cryosections (8 μ m) were mounted on glass slides, and hematoxylin and eosin and sirius red staining were performed as described.⁽¹⁵⁾ Immunostaining was performed by cold acetone fixation. The fixed sections were incubated with ImmunoBlock (DS Pharma Biomedical) and then incubated with Ly6G (BioLegend) or F4/80 (BioLegend) and collagen I (Bio-Rad, Hercules, CA) or *Mmp9* (Abcam) antibodies, followed by Alexa555-labeled anti-rat and Alexa488-labeled anti-rabbit secondary antibodies (Molecular Probes). Images were captured using Observer Z1 with an AxioCam HRc (Zeiss, Oberkochen, Germany) except for quantification of sirius red-positive areas, which were analyzed using cellSens software (Olympus, Tokyo, Japan). Ly6G-positive⁽⁺⁾ or F4/80⁺ cell

TABLE 1. PRIMER SEQUENCES USED FOR THIS STUDY

For qPCR:

Actb; 5'-ccaaccgtgaaaagatgacc-3', 5'-ccagaggcatacagggacag-3'
Cyp2e1; 5'-ccaccagcacaactctgagata-3', 5'-cccaataaccctgtcaatttctt-3'
Acta2; 5'-gacaccaccccccagagt-3', 5'-acatagctggagcagcgtct-3'
Tgfb1; 5'-tcagacattcgggaagcagt-3', 5'-acgccaggaattgttctat-3'
Timp1; 5'-gcaaaagagctttctcaagacc-3', 5'-aggatagataaacagggaaacct-3'
Mmp8; 5'-cactccgtggggagattac-3', 5'-aagaccgttgggtaggaagg-3'
Mmp9; 5'-tgactacgataaggacggcaaa-3', 5'-agatgaacgggaacacacagg-3'
Mmp13; 5'-ctggcacacgcttttctc-3', 5'-atgggcagcaacaataaacaag-3'
Cxcl1; 5'-ggattcacctcaagaacatccag-3', 5'-atctttggacaattttctgaacc-3'
Pdgfrb; 5'-cggcctgtgactagaagtc-3', 5'-gagcttgaggcgtcttgg -3'
Il1b; 5'-agttgacggaccccaaaag -3', 5'-agctggatgctctcatcagg -3'
Il17a; 5'-tgtgaaggtaacctcaagtc -3', 5'-gagggatctatcagggtctca -3'
Cxcl2; 5'-ccaaaagatctgaacaaaggcaag -3', 5'-ggcacatcaggtacatcca -3'

For expression vector construction:

Cre; 5'-agcaattcgcgcaccatgggtgccaa -3', 5'-gtgatctcagtcctccatctc -3'
Cxcl1; 5'-gaattcatgatccaccagcccgctcgc-3', 5'-aagctttacttgggacaccttttagc-3'
Mmp8; 5'-gaattcatgttgcctgaagacactccattac-3', 5'-cccgggctatgaacagtttaagccataaattgc-3'
Mmp9; 5'-gaattcatgatcctcctggcagccctgctcctggc-3', 5'-aagctcaaggcactgcaggaggtctaggtc-3'

numbers were counted using Image J software (for Figs. 2–5). Ly6G and MMP9 double-positive cell numbers were counted using the BZ-X700 hybrid cell count software (Keyence, Osaka, Japan).

WESTERN BLOTTING

Whole liver lysates were used for western blot analysis. Liver samples were sonicated in ice-cold lysis buffer containing 10 mM CaCl₂ and 0.2% Triton X-100. We used liver microsomal fractions for Cyp2e1 analysis. The lysate was centrifuged at 10,000g for 10 minutes at 4°C, the supernatant was collected, and the protein concentration was determined by the Lowry assay (Bio-Rad). Twenty micrograms of total protein extract was loaded onto a 10% polyacrylamide gel, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred to a Immobilon-P polyvinylidene fluoride membrane (Millipore). After washing with trishydroxymethylaminomethane-buffered saline containing 5% skimmed milk and 0.2% Tween 20, the membranes were incubated with antibody against α -smooth muscle actin (α SMA; 1:400), actin (1:1,000), Mmp8 (1:2,000), Mmp9 (1:1,000), or Cyp2e1 (1:5,000) overnight at 4°C.

After washing with trishydroxymethylaminomethane-buffered saline Tween 20, the membranes were probed with a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Little Chalfont, United Kingdom), and immunodetection was performed with a Western Lightning Plus enhanced chemiluminescence reagent (Perkin Elmer). Following immunodetection,

membranes were incubated in Coomassie Brilliant blue as a loading control for protein quantification and signals were quantified with a luminoimage analyzer (LAS 4100; Fuji Film, Tokyo, Japan). Band intensity was measured using the Image J software.

GELATIN ZYMOGRAPHY

MMP9 activity of whole liver was detected with a gelatin-zymography kit (Primary Cell, Sapporo, Japan) according to the manufacturer's instructions. In brief, each sample (20 μ g), identical to that used for western blot analysis, was loaded for electrophoresis. Gels were washed and incubated 20 hours in incubation buffer (attached with kit) at 37°C, stained with Coomassie Brilliant blue, and scanned.

RT-PCR AND REAL-TIME PCR

Total RNA was isolated from mouse liver or blood cells using TRIzol reagent (Invitrogen). RT to cDNA templates was performed using PrimeScript RT Master Mix (Takara Bio, Kyoto, Japan). Real-time RT-PCR experiments were conducted with a LightCycler 96 system (Roche Diagnostics, Indianapolis, IN). The mouse actin beta gene assay was used as the normalization control. Sequence information for the primer pairs used is listed in Table 1.

CELL ISOLATION

A single-cell suspension was obtained from livers by the collagenase perfusion method as described.⁽¹⁵⁾

Liver macrophages and neutrophils and bone marrow neutrophils were sorted by AutoMACS (Miltenyi Biotec) using the repeated Possel program.

QUANTITATIVE ANALYSIS OF LIVER SECTIONS STAINED WITH SIRIUS RED

Fibrotic areas detected by sirius red-stained collagen fibers were quantified in the liver sections at 20× magnification. The mean value of 21 serial areas per sample was used as the percentage area of fibrosis (cellSens software; Olympus).

FLOW CYTOMETRY

Liver nonparenchymal cells were obtained by liver perfusion and dissociation to assess liver blood cell populations. For flow cytometric analysis, propidium iodide was used for staining dead cells and antibodies (BioLegend) were used to detect clusters of differentiation (CD)45, CD11b, F4/80, Ly6G, CD68, and CD206. To evaluate donor chimerism, peripheral blood samples were collected from the recipients after transplantation. After lysis of erythrocytes, cells were stained with propidium iodide and CD45.1 and CD45.2 antibodies. Donor contribution was calculated as follows: percentage of donor chimerism = $(100 \times \text{CD45.1 cells} [\%]) / (\text{CD45.1 cells} [\%] + \text{CD45.2 cells} [\%])$.

NEUTROPHIL DEPLETION BY LY6G-SPECIFIC ANTIBODY

Neutrophil depletion was performed as described⁽¹⁶⁾ with slight modifications. In brief, 500 μg of purified anti-Ly6G rat monoclonal antibody (1A8; BioLegend) was administered to mice 6 hours before and 48 hours after the final CCl₄ administration. Control animals received a similar dose of unlabeled rat immunoglobulin G (Beckman Coulter). Depletion of neutrophils in the peripheral blood was confirmed by fluorescence-activated cell sorting (FACS) analysis.

MICROSOMAL PREPARATION

Liver microsomes were prepared as described.⁽¹⁷⁾ In brief, livers were homogenized in cold 0.15 M KCl and 10 mM ethylene diamine tetraacetic acid (pH 8.0). Following centrifugation of the homogenate at 15,000g for 15 minutes, microsomes were pelleted from the supernatant fraction by centrifugation at 105,000g for 60 minutes.

DETERMINATION OF Cyp2e1 ACTIVITY

Cyp2e1 activity was determined as described.⁽¹⁸⁾ We used 100 μg of microsomal samples per reaction individually.

SERUM LEVELS OF ALBUMIN, ASPARTATE AMINOTRANSFERASE, AND ALANINE AMINOTRANSFERASE

Albumin, aspartate aminotransferase, and alanine aminotransferase (ALT) in sera were measured by Oriental Yeast (Tokyo, Japan).

STATISTICAL ANALYSIS

Statistical analysis was performed using the unpaired two-tailed Student *t* test. $P < 0.05$ was considered statistically significant.

Results

REDUCED FIBROSIS AND INCREASED EXPRESSION OF NEUTROPHIL MMPs IN *Trib1* KO LIVER

Loss of *Trib1* was previously shown to decrease tissue-resident M2-like macrophages and eosinophils and increase neutrophils by altering the expression level of C/EBP α in an E3-ubiquitin ligase constitutive photomorphogenic 1-dependent manner.⁽¹²⁾ Because macrophages have been implicated in liver fibrosis, we investigated liver fibrosis in *Trib1*-deficient mice. After repeated administration of CCl₄ for 4 weeks, sirius red staining of the liver sections revealed that collagen accumulation in *Trib1* KO liver was significantly reduced compared with that of wild-type littermates (Fig. 1A,B). Cyp2e1 is the major enzyme responsible for CCl₄-induced liver injury,⁽¹⁹⁾ but its expression level was not affected by loss of *Trib1* (Fig. 1C). As continuous administration of CCl₄ transforms quiescent HSCs to myofibroblasts that produce ECM, we examined expression of key fibrogenic cytokines *Tgfb1* and actin $\alpha 2$ (*Acta2*), which are activation markers of myofibroblasts. However, there was no significant difference in the expression of these markers in wild-type and *Trib1* KO liver after 4 weeks of continuous CCl₄

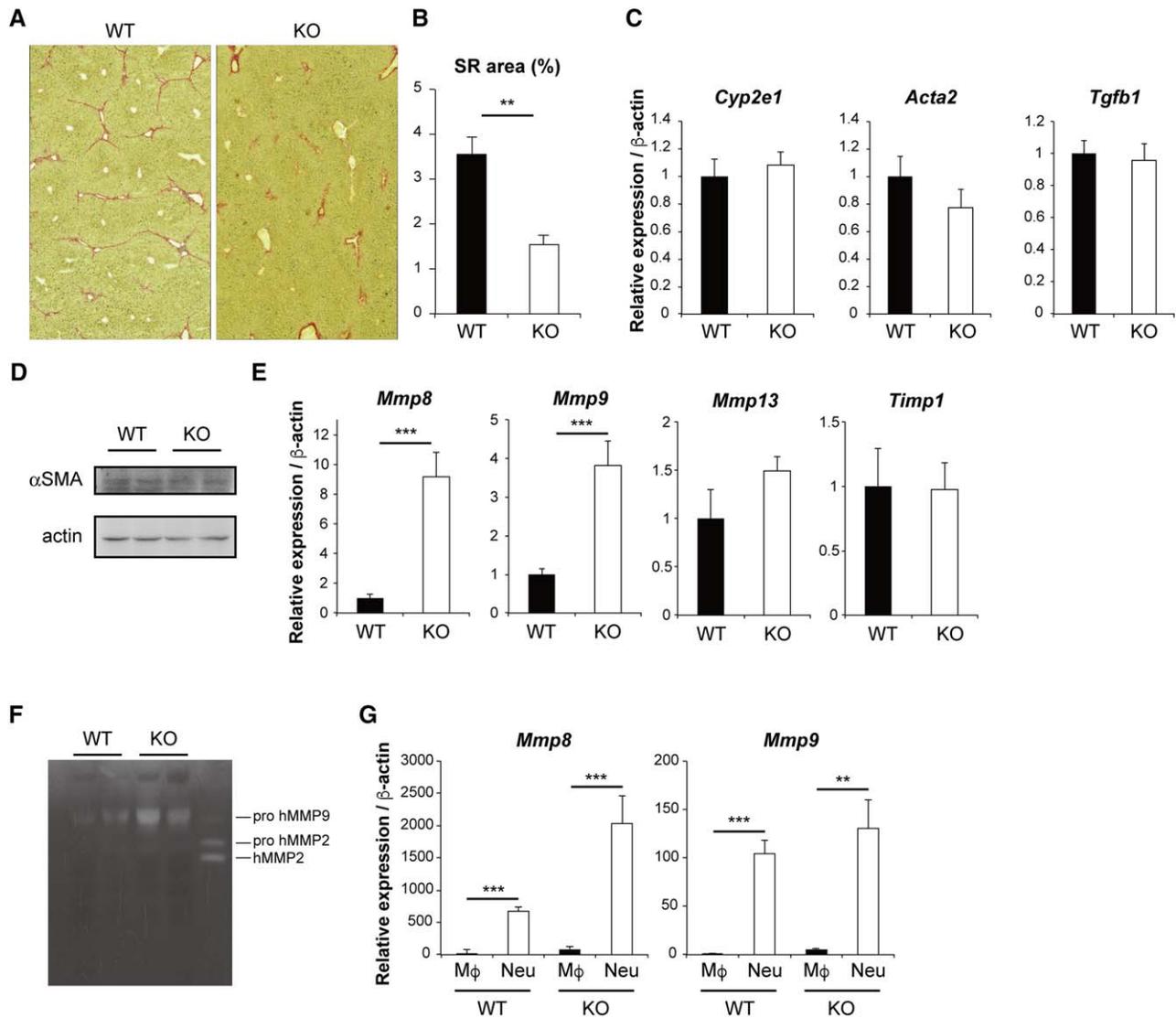


FIG. 1. *Trib1*-deficient mice exhibit reduced liver fibrosis and increased expression of neutrophil *Mmps*. (A) Histochemical staining for collagen. CCl₄ was injected into each mouse 2 times per week for 4 weeks; the liver was harvested 72 hours after the final CCl₄ administration. Sirius red staining of liver sections after CCl₄ treatment. These pictures were represented by 21 serial tiling images (each magnification $\times 20$). (B) Quantification of collagen-positive area in liver sections as shown in (A) (WT, n = 10, *Trib1* KO, n = 7 per group). (C) Real-time RT-PCR analysis of *Cyp2e1*, *Acta2*, and *Tgfb1* in whole liver cDNA (n = 7 per group). (D) Western blot analysis for α SMA of extracts from wild-type littermates and *Trib1* KO liver. Actin was used for the loading control. (E) Real-time RT-PCR analysis of *Mmp8*, *Mmp9*, *Mmp13*, and *Timp1* in whole liver cDNA (n = 7 per group). (F) Gelatin-zymography of extracts from wild-type and *Trib1* KO liver after chronic CCl₄ administration. (G) Real-time RT-PCR analysis of *Mmp8* and *Mmp9* in macrophages or neutrophils. Macrophages were isolated from normal liver by collagenase perfusion followed by magnetic bead sorting with F4/80 antibody. Neutrophils were isolated from bone marrow by magnetic bead sorting with Ly6G antibody (n = 3 per group). Data are expressed as means \pm SEM. ***P* < 0.01, ****P* < 0.001. Abbreviations: M ϕ , macrophage; Neu, neutrophil; pro hMMP, pro-form human MMP; SR, sirius red; WT, wild type.

administration (Fig. 1C). Although *Cyp2e1* protein levels and enzymatic activity in liver were slightly low in *Trib1* KO liver (Supporting Fig. S1E,F), necrotic area and expression levels of *Tgfb1* and *Acta2*/ α SMA in *Trib1* KO mice were comparable to those in wild-type littermates (Supporting Fig.

S1A-D). Protein expression levels of α SMA were also comparable between wild-type and *Trib1* KO liver (Fig. 1D). Because lack of *Trib1* did not cause changes to the expression of *Acta2* and *Tgfb1*, we hypothesized that fibrolysis may be altered in *Trib1*-deficient mice.

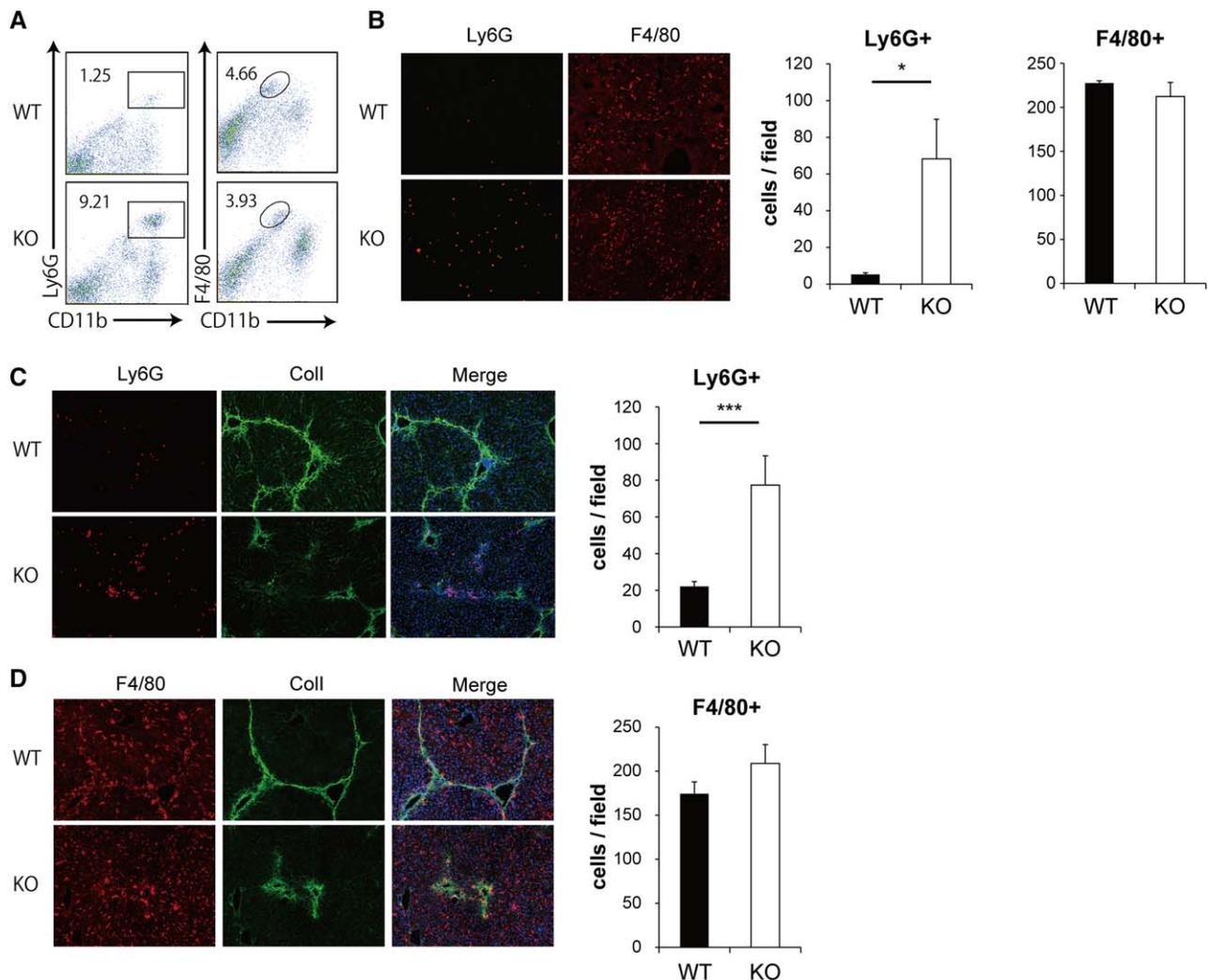


FIG. 2. Increased liver-resident neutrophils in *Trib1* KO mice. (A) Flow cytometric analysis of nonparenchymal cells from untreated liver. Numbers indicate the percentage of cells in the gate. The CD11b⁺ F4/80⁺ macrophage population was slightly reduced but the CD11b⁺ Ly6G⁺ neutrophil population was dramatically increased in *Trib1* KO liver. (B) Immunohistochemical staining for Ly6G or F4/80 in untreated liver. Graphs show Ly6G⁺ or F4/80⁺ cell numbers in each field. Counting of Ly6G⁺ or F4/80⁺ cells was carried out with Image J analysis software (n = 4 per group; magnification $\times 10$). (C,D) Mice were treated with CCl₄ 2 times per week for 4 weeks; this was followed by harvesting of the liver. Immunostaining for (C) Ly6G (red) or (D) F4/80 (red) and collagen type 1) (green) in liver. Graphs show Ly6G⁺ or F4/80⁺ cell numbers in each field (n = 7 per group; magnification $\times 10$). Data are expressed as means \pm SEM. * $P < 0.05$, *** $P < 0.001$. Abbreviations: Coll, collagen type 1; WT, wild type.

As MMPs are critically involved in the resolution of fibrosis,⁽¹⁾ we examined the expression of *Mmps* and found that *Trib1*-deficient liver exhibited a marked increase of *Mmp8* (collagenase) and *Mmp9* (gelatinase B) whereas the expression level of *Mmp13*, an interstitial collagenase, was unchanged (Fig. 1E). Furthermore, expression of tissue inhibitor of MMP-1 (*Timp1*), a negative regulator of MMPs, was comparable between wild-type and *Trib1* KO

liver. Protein levels of MMP8 and MMP9 were also increased in *Trib1* KO liver lysate (Supporting Fig. S4A,B). Increased MMP9 enzymatic activity in *Trib1* KO liver was confirmed by zymography (Fig. 1F). These results strongly suggest that increased MMP activity in *Trib1* KO liver is responsible for the reduced fibrosis. MMP8 is a known neutrophil collagenase, and MMP9 is produced by myeloid cells.⁽²⁰⁾ To identify the cell source of MMPs, we

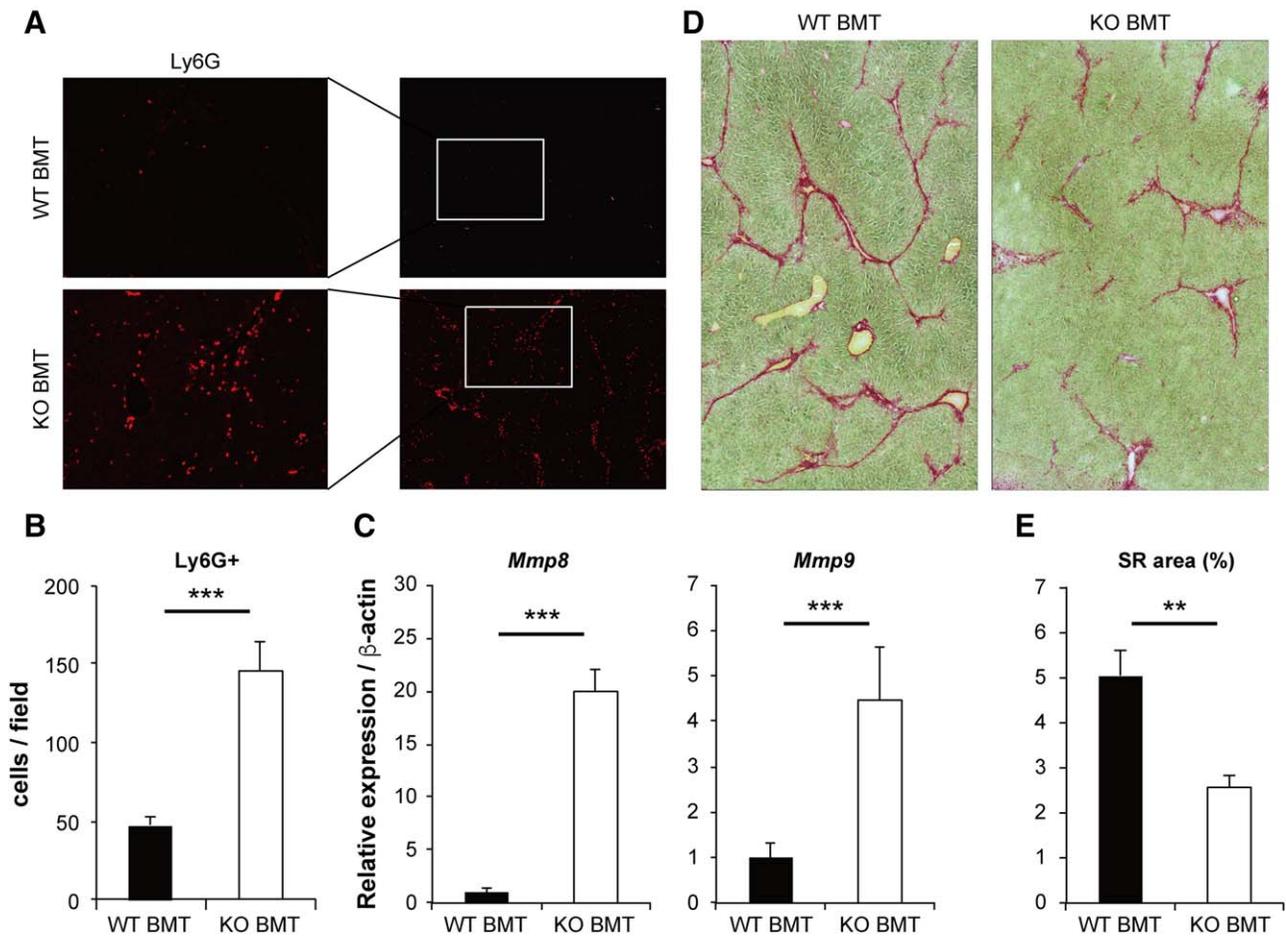


FIG. 3. Suppression of liver fibrosis by transplantation of *Trib1* KO bone marrow cells. Ly5.1 mice received wild-type or *Trib1* KO bone marrow transplants following busulfan treatment. CCl₄ was injected into each mouse 2 times per week for 4 weeks; this was followed by harvesting of the liver. (A) Immunostaining for Ly6G in liver sections. (B) Ly6G⁺ cell numbers in each field (WT BMT, n = 6; KO BMT, n = 5; magnification $\times 10$). (C) Expression levels of *Mmp8* and *Mmp9* in whole liver (WT BMT, n = 6; KO BMT, n = 5). (D) Histochemical staining for collagen in liver sections. Photomicrographs illustrate hepatic collagen fibrils stained with sirius red. These pictures were represented by 21 serial tiling images (each magnification $\times 20$). (E) Quantification of collagen-positive area in liver sections as shown in (C) (WT BMT, n = 6; KO BMT, n = 5). Data are expressed as means \pm SEM. ** $P < 0.01$, *** $P < 0.001$. Abbreviations: BMT, bone marrow transplant; SR, sirius red; WT, wild type.

sorted Ly6G⁺ neutrophils and F4/80⁺ macrophages and examined expression levels of *Mmps* by qPCR (Fig. 1G). Results showed that the major source of *Mmp8* and *Mmp9* was neutrophils rather than macrophages. We also performed co-immunofluorescence staining for Ly6G and MMP9 to identify localization of the cells and matrix-degrading activity (Supporting Fig. S5). Although Ly6G⁺ cell numbers were significantly different between wild-type and *Trib1* KO liver (Fig. 2), the MMP9 signal was colocalized with Ly6G in both genotypes. These results indicate that loss of *Trib1* increases the expression of neutrophil-derived MMPs in the liver.

NEUTROPHILS ARE ABUNDANT IN *Trib1* KO LIVER

To address whether the up-regulation of *Mmps* in *Trib1* KO liver is due to the increased number of neutrophils in the mutant liver, we examined Ly6G⁺ cells and F4/80⁺ cells by FACS. Even before the administration of CCl₄, Ly6G⁺ neutrophils were clearly increased in *Trib1* KO liver whereas F4/80⁺ macrophages were slightly reduced, consistent with a previous report⁽¹²⁾ (Fig. 2A; Supporting Fig. S6). Immunohistochemical analysis also showed that the Ly6G⁺ neutrophil population was increased in *Trib1* KO liver whereas the F4/80⁺

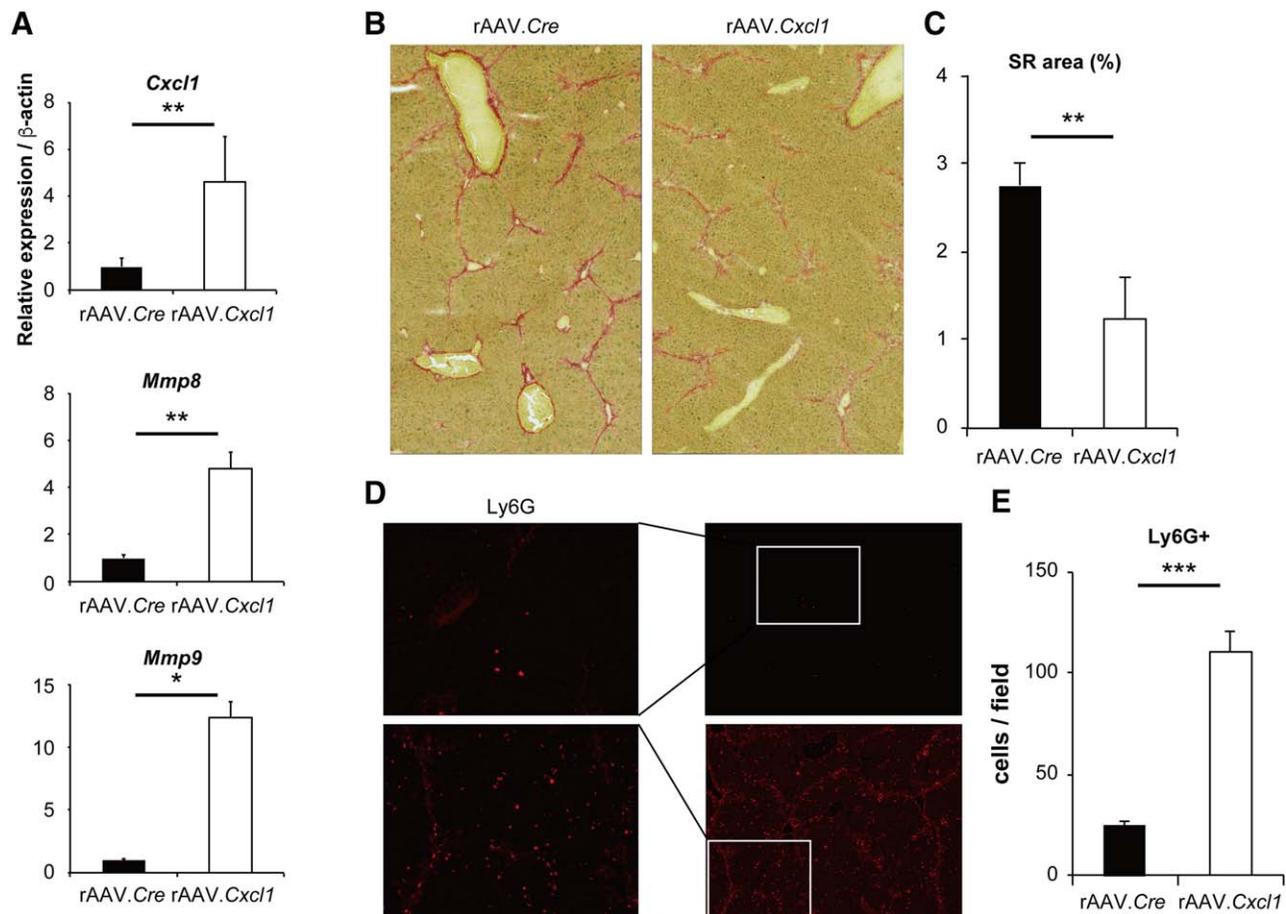


FIG. 4. Recruitment of neutrophils to the liver suppresses fibrosis. *Cxcl1* was expressed in liver by administration of rAAV.*Cxcl1* (1×10^{11} vg per mouse) 2 weeks before CCl₄ administration; CCl₄ was then administered for 4 weeks. (A) Expression levels of *Cxcl1*, *Mmp8*, and *Mmp9* in whole liver (n = 6 per group). (B) Collagen accumulation in the liver treated with CCl₄ for 4 weeks. Photomicrographs illustrate hepatic collagen fibrils stained with sirius red. These pictures were represented by 21 serial tiling images (each magnification $\times 20$). (C) Quantification of collagen-positive area in liver sections as shown in (B) (n = 6 per group). (D) Immunostaining for Ly6G in liver sections treated with CCl₄ for 4 weeks (magnification $\times 10$). (E) Ly6G⁺ cell numbers in each field (n = 6 per group). Data are expressed as means \pm SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Abbreviation: SR, sirius red.

macrophage population was slightly decreased in *Trib1* KO liver (Fig. 2B). After 4 weeks of CCl₄ administration, the Ly6G⁺ cells were more abundant in *Trib1* KO liver compared with wild-type littermates whereas F4/80⁺ cells were not increased in *Trib1* KO liver (Fig. 2C,D). These results indicate that neutrophils are abundant in *Trib1* KO liver before and after CCl₄ administration.

TRANSPLANTATION OF *Trib1* KO BONE MARROW CELLS ALLEVIATES LIVER FIBROSIS

To rule out the possible influence of altered expression of hepatocytic metabolic enzymes on the *Trib1* KO

phenotype, we investigated whether blood cells, including neutrophils, are responsible for the suppression of liver fibrosis. For transplantation of *Trib1* KO bone marrow cells, busulfan was administered to pregnant Ly5.1 congenic mice to deplete bone marrow cells. Busulfan-treated neonatal mice were transplanted with wild-type or *Trib1* KO bone marrow cells. We used chimeric mice that showed at least 70% donor chimerism in the peripheral blood (Supporting Fig. S7). Transplantation of *Trib1* KO bone marrow cells but not wild-type littermate cells increased Ly6G⁺ cells in the liver (Fig. 3A,B). Consistently, *Trib1* KO bone marrow cells increased *Mmps* expression in liver after CCl₄ treatment (Fig. 3C). Furthermore, sirius red staining showed that collagen deposition was significantly decreased by transplantation of

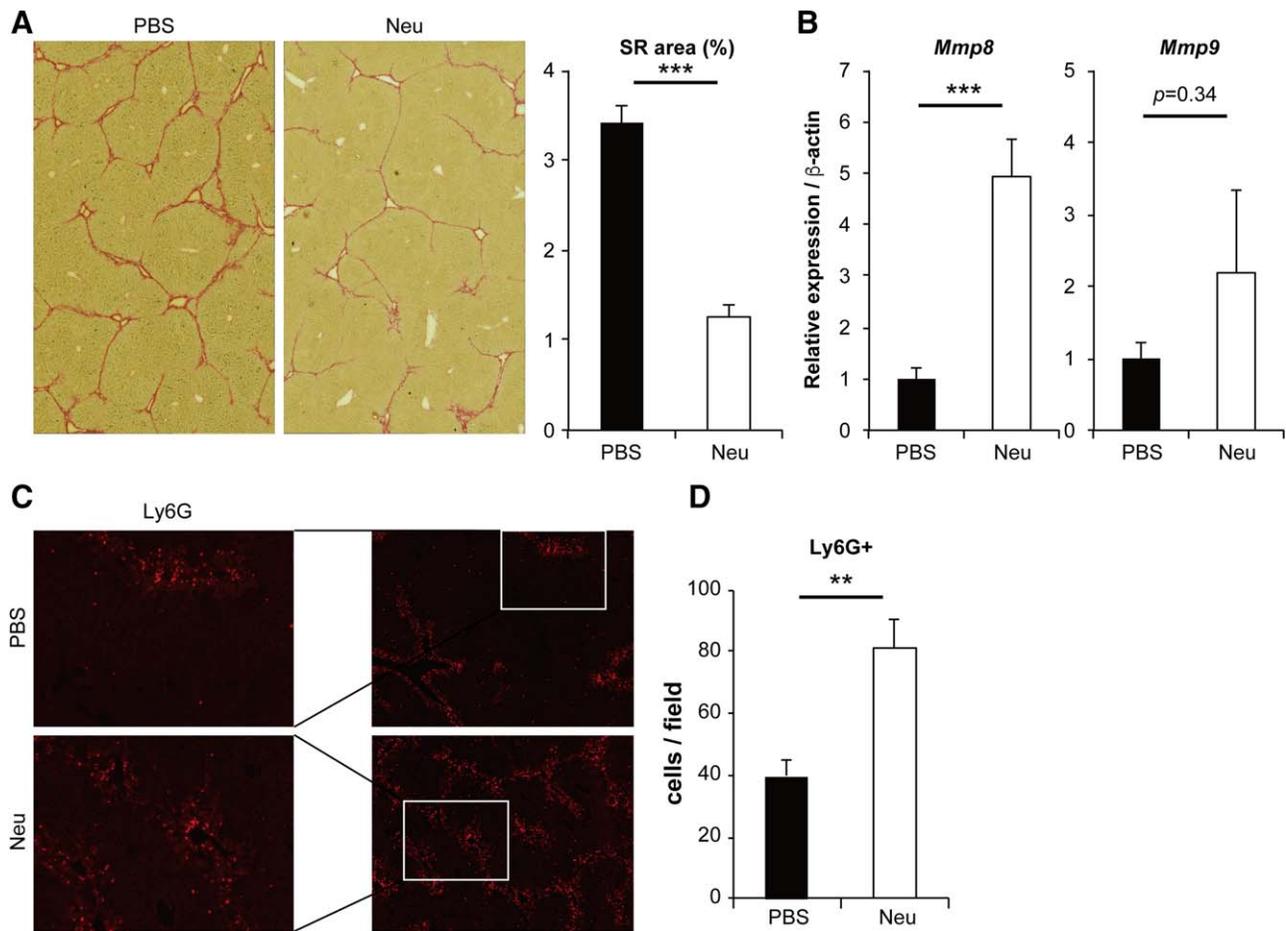


FIG. 5. Infusion of wild-type neutrophils suppresses CCl₄-induced liver fibrosis. Mice were treated with CCl₄ 2 times per week for 4 weeks; this was followed by injection of bone marrow-derived neutrophils (5×10^6 cells/mouse) into the tail vein. PBS indicates control animals injected with PBS and Neu indicates neutrophil-infused mice. (A) Sirius red staining of collagen in liver (left). Quantification of collagen-positive area in liver sections (right) (n = 6 per group; ***P < 0.001). These pictures were represented by 21 serial tiling images (each magnification $\times 20$). (B) Expression levels of *Mmp8* and *Mmp9* in whole liver (n = 6 per group; ***P < 0.001). (C) Mice were treated with CCl₄ for 4 weeks followed by injection of 5×10^6 cells of bone marrow-derived neutrophils. Neutrophil (red) in the liver at 1 hour after neutrophil infusion (magnification $\times 10$). (D) Ly6G⁺ cell numbers in each field (PBS, n = 4; Neu, n = 5). Data are expressed as means \pm SEM. **P < 0.01. Abbreviations: Neu, neutrophil; PBS, phosphate-buffered saline; SR, sirius red.

Trib1 KO bone marrow cells (Fig. 3D,E). These data demonstrate that blood cells derived from *Trib1* KO bone marrow cells alleviated CCl₄-induced liver fibrosis.

rAAV-MEDIATED EXPRESSION OF *Cxcl1* IN WILD-TYPE LIVER RECRUITS NEUTROPHILS AND SUPPRESSES LIVER FIBROSIS

To address whether wild-type neutrophils are capable of suppressing fibrosis as effectively as *Trib1* KO neutrophils, we expressed *Cxcl1* (also known as KC), a

neutrophil chemoattractant chemokine, by rAAV vector under the control of a liver-specific promoter.⁽¹⁴⁾ Adeno-associated virus serotype 8 was chosen as it can efficiently transduce hepatocytes in mice. Induction of *Cxcl1* expression in the wild-type liver resulted in a marked increase in the number of neutrophils in the liver without inducing liver injury as evidenced by unchanged levels aspartate aminotransferase/ALT (Supporting Fig. S8).

We then administered CCl₄ 8 times after transduction with rAAV.*Cxcl1*. Following CCl₄ administration, the expression of *Cxcl1* in the liver was confirmed (Fig. 4A). Consistent with increased expression of *Cxcl1*,

expressions of *Mmp8* and *Mmp9* were significantly increased in liver that received rAAV.*Cxcl1* (Fig. 4A). The number of Ly6G⁺ cells was also significantly increased in rAAV.*Cxcl1* transduced liver compared to those that received the control vector rAAV.*Cre* (Fig. 4D,E), and sirius red staining was reduced by *Cxcl1* expression (Fig. 4B,C), indicating that accumulation of neutrophils in the liver alleviates liver fibrosis.

INFUSION OF WILD-TYPE NEUTROPHILS ALLEVIATES CCl₄-INDUCED LIVER FIBROSIS

To further examine the beneficial effect of neutrophils in injured liver, we adoptively transferred 5×10^6 normal bone marrow neutrophils into mice that received CCl₄ for 4 weeks. Neutrophils were isolated using magnetic activated cell sorting with 14 kinds of antibodies to deplete blood cells except for neutrophils. Consistent with the above findings, infusion of neutrophils significantly reduced collagen accumulation in the liver within 2 days after infusion (Fig. 5A). In contrast, infusion of the Ly6G-negative cell population did not reduce liver fibrosis (Supporting Fig. S9). These results indicate that infused neutrophils effectively reduced fibrosis. Notably, as the average lifespan of mouse neutrophils is estimated to be 1.5–12.5 hours in tissue,⁽⁷⁾ transferred neutrophils were not detected at the time of sacrifice. Thus, to verify whether transferred neutrophils reached the liver, we killed mice 1 hour after infusion. Immunohistochemical staining of liver sections revealed that the Ly6G⁺ cells were significantly increased in mice that had been infused with neutrophils (Fig. 5C,D). Furthermore, the expression levels of *Mmp8* and *Mmp9* were also increased (Fig. 5B). These results suggest that adoptively transferred neutrophils rapidly migrate to the liver and resolve collagen fibers.

EXPRESSION OF *Mmp8* AND *Mmp9* AMELIORATES CCl₄-INDUCED LIVER FIBROSIS

We next investigated the mechanisms of neutrophil-mediated resolution of fibrosis. Because the expression of *Mmp8* and *Mmp9* is a characteristic feature of neutrophils, we transduced both *Mmp8* and *Mmp9* cDNA into the liver by rAAV vector after repeated treatments of CCl₄ for 6 weeks. The expression levels of *Mmps* were verified by qRT-PCR (Fig. 6A). Because we fixed the total dose to 1×10^{11} vector genomes (vg) of

rAAV per mouse, for the combinatorial expression of both *Mmps*, mice received a dose of 5×10^{10} vg for each vector. Expression of *Mmp8* or *Mmp9* resulted in a marked decrease in collagen staining by sirius red (Fig. 6B,C). Serum ALT levels showed no significant hepatic damage by overexpression of *Mmp8* and *Mmp9* (Fig. 6D). Thus, these results suggest that the resolution of liver fibrosis by neutrophils can be, at least in part, due to expressions of *Mmp8* and *Mmp9*. Taken together, these findings indicate that neutrophils play a beneficial role in the resolution of liver fibrosis.

NEUTROPHIL IMMUNE DEPLETION EXACERBATES CCl₄-INDUCED LIVER FIBROSIS

We depleted neutrophils by Ly6G antibody (1A8) to further prove that neutrophils play a role for resolution of liver fibrosis. According to the protocol described,^(21–23) we administrated 500 μg of antibody twice (Fig. 7A). FACS analysis showed that the Ly6G⁺CD11b⁺ cells were markedly decreased at 72 hours after the final CCl₄ administration when the scar resolution was most active (Fig. 7B).⁽⁶⁾ Consistently, immunostaining of liver sections demonstrated a significant decrease of Ly6G⁺ cells in liver at 96 hours after the final CCl₄ administration (Fig. 7C–F). Moreover, sirius red staining analysis clearly showed that fibrosis resolution was significantly delayed by anti-Ly6G antibody treatment (Fig. 7G,H). These findings indicate that neutrophils are involved in natural fibrosis resolution in liver.

Discussion

In this study, we showed that loss of *Trib1* results in the accumulation of neutrophils in the liver, leading to reduced fibrosis in the chronic liver injury model induced by CCl₄. We also showed that recruiting neutrophils by rAAV-mediated expression of *Cxcl1* in the liver as well as infusion of neutrophils alleviated fibrosis. *Trib1*-deficient liver exhibited increased expression of the neutrophil proteases *Mmp8* and *Mmp9*, and their rAAV-mediated expression in liver reduced CCl₄-induced fibrosis. Neutrophil depletion by Ly6G antibody delayed liver fibrosis resolution (Fig. 7). Taken together, these results demonstrate that neutrophils play a beneficial role for CCl₄-induced liver fibrosis by producing these MMPs.

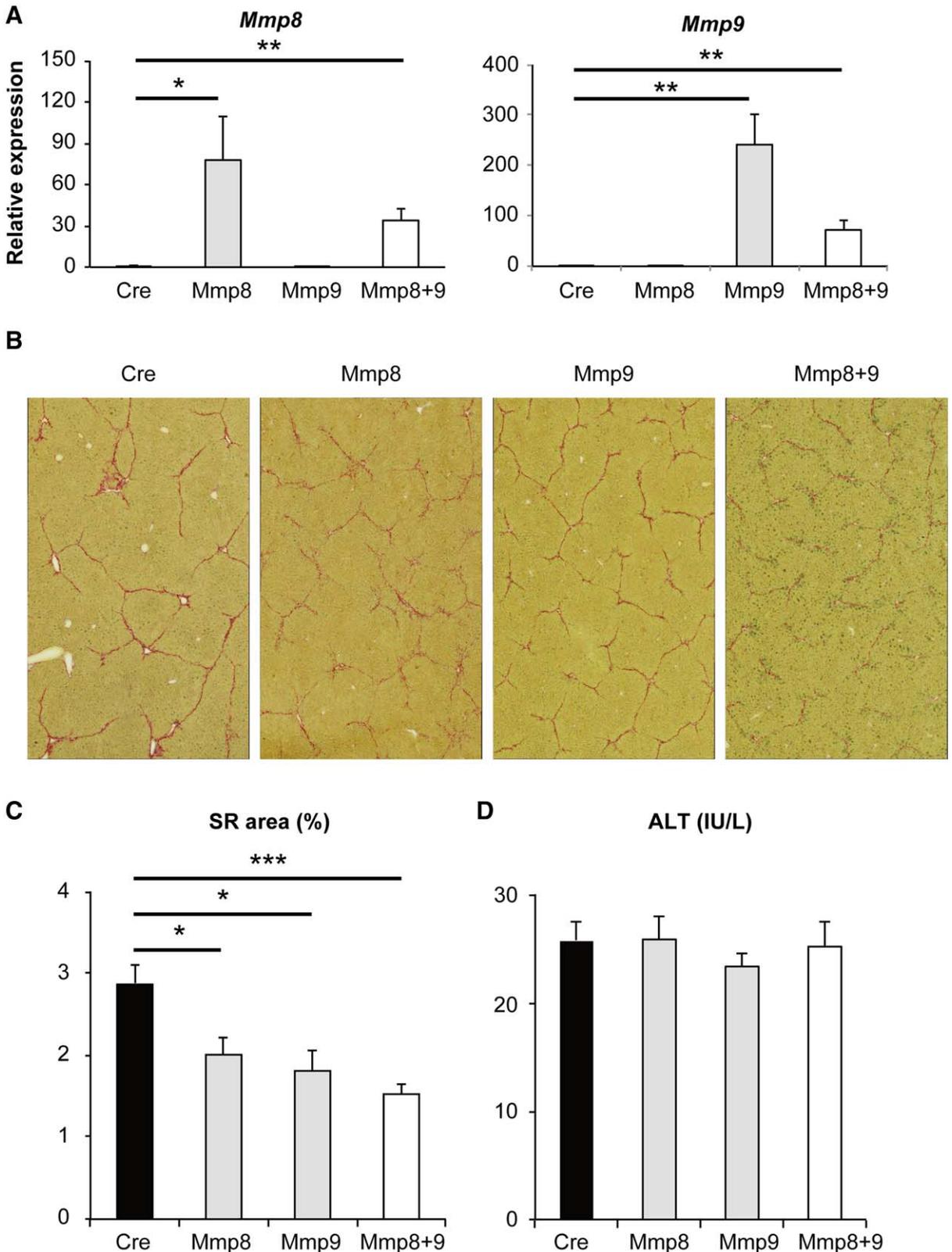


FIG. 6. Hepatic expression of *Mmp8* and *Mmp9* reduces CCl_4 -induced liver fibrosis. Mice were treated with CCl_4 2 times per week for 6 weeks; this was followed by injection with rAAV.*Mmp8* or rAAV.*Mmp9* to express *Mmp8* and *Mmp9* for 2 weeks. For expression of *Mmp8* and *Mmp9* alone, a dose of 10^{11} vg of rAAV was injected intraperitoneally into each mouse. For combined expression of *Mmp8* and *Mmp9*, a dose of 5 ± 10^{10} vg was used for each vector, resulting in a combined dose of 10^{11} vg of rAAV per mouse. (A) Expression levels of *Mmp8* and *Mmp9* in whole liver ($n = 6$ per group). (B) Sirius red staining for collagen in liver 2 weeks after rAAV transduction. These pictures were represented by 21 serial tiling images (each magnification $\times 20$). (C) Quantification of collagen-positive area in murine liver sections as shown in (B) ($n = 6$ per group). (D) Evaluation of serum ALT levels at 2 weeks after rAAV vector administration ($n = 6$ per group). Data are expressed as means \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Abbreviation: SR, sirius red.

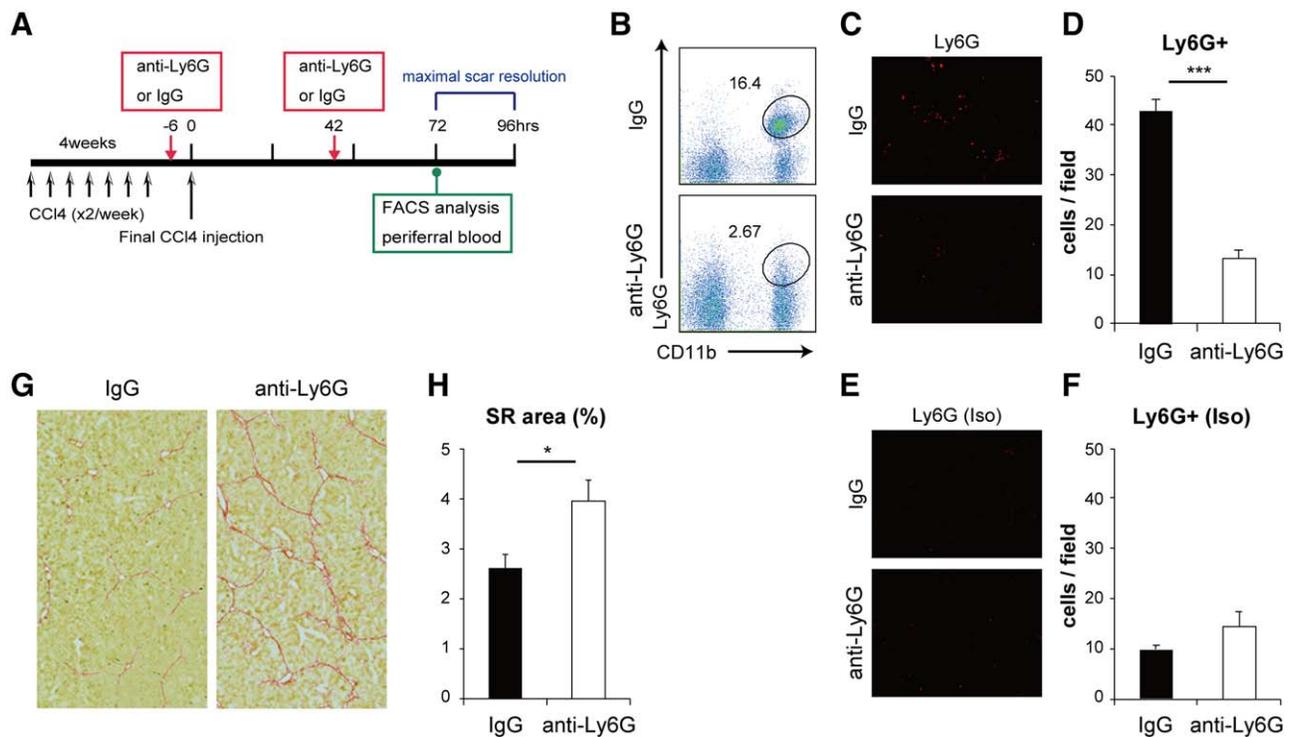


FIG. 7. Immunodepletion of neutrophils enhances CCl₄-induced liver fibrosis. (A) Experimental design. Hepatic fibrosis was induced in C57BL/6 mice by intraperitoneal injection of CCl₄ 2 times per week for 4 weeks. Next, 500 μ g of Ly6G (1A8) antibody or control IgG was administered intraperitoneally 6 hours before the final injection and 42 hours after the final injection of CCl₄. (B) Flow cytometric analysis of the peripheral blood at 72 hours after the final CCl₄ injection. Numbers indicate the percentage of cells in the gate. The CD11b⁺Ly6G⁺ neutrophil population was dramatically reduced by immunodepletion. (C,D) Immunohistochemical staining for Ly6G in liver. Ly6G⁺ cell numbers in each field were determined using Image J analysis software (IgG, n = 7; anti-Ly6G, n = 6; magnification \times 10). (E,F) Isotype control of (C,D). (G) Collagen accumulation in the liver treated with CCl₄ for 4 weeks. Tissues were harvested at 96 hours after the final CCl₄ injection. Photomicrographs illustrate hepatic collagen fibrils stained with sirius red. These pictures were represented by 21 serial tiling images (each magnification \times 20). (H) Quantification of collagen-positive area in liver sections shown in (G) (IgG, n = 7; anti-Ly6G, n = 6). Data are expressed as means \pm SEM. * P < 0.05, *** P < 0.001. Abbreviations: IgG, immunoglobulin G; SR, sirius red.

Because M2 macrophages have been considered to be profibrotic by producing TGF- β and lack of *Trib1* was previously shown to decrease tissue resident M2-like macrophages and increase neutrophils,⁽¹²⁾ we hypothesized that liver fibrosis would be altered in *Trib1* KO mice. Although macrophages in *Trib1* KO liver were slightly decreased, no significant change was observed in the expression of fibrogenic TGF- β at 48 hours after either a single dose or continuous administration of CCl₄ (Supporting Fig. S1C; Fig 1C). Instead we found a significant increase of neutrophils in *Trib1* KO liver. As *Trib1* is a negative regulator of C/EBP α and C/EBP α is required for neutrophil differentiation from myeloid progenitors, the increase of neutrophils in *Trib1* KO mice was likely due to a consequence of up-regulation of C/EBP α , consistent with reports showing that overexpression of C/EBP α increased the neutrophil

population.⁽¹¹⁾ There was no statistically significant difference in expression of neutrophil cytokines in *Trib1* KO liver (Supporting Fig. S2), suggesting that accumulation of neutrophils in the liver was due to increased neutrophil production rather than increased neutrophil chemoattractants in the liver.

Neutrophils are the most abundant leukocytes in the circulatory system and have been considered to play key roles in acute inflammatory responses, whereas the roles of neutrophils in chronic inflammation have not been intensively studied. In many tissues, neutrophils are recruited to the injury site and promote inflammatory reactions. Likewise, it has been well established that neutrophils are rapidly recruited to the liver in acute liver injury models and exacerbate inflammatory responses, leading to occasional liver failure.⁽⁸⁾ However, the role of neutrophils in chronic hepatitis

remains uncertain. The most interesting finding of our study is that neutrophils play an antifibrotic role in chronic liver injury. As fibrosis was ameliorated by recruiting wild-type neutrophils by rAAV-mediated expression of *Cxcl1* in the liver and also by infusion of wild-type neutrophils, it is likely that the effect of *Trib1* KO was due to the increased number but not functional alteration of neutrophils by the mutation.

Neutrophils reportedly have a minimal impact on wound repair and regeneration,⁽²²⁾ which contradicts our findings. However, the experimental setting of Moles et al. differed from ours in that they used S100A9 KO mice in which inflammatory cytokines were reduced and neutrophil levels in the liver remained 50%-60% of the normal level.⁽²²⁾ In our system, neutrophil levels were reduced to 14% in the peripheral blood and 30% in the liver by Ly6G antibody. Thus, we think that neutrophil levels in the liver could be a reason for the difference.

The beneficial role of neutrophils for liver injuries is not completely unexpected. Infusion of bone marrow-derived macrophages has been shown to reduce hepatic fibrosis by recruiting endogenous macrophages and neutrophils.⁽³⁾ Administration of granulocyte colony-stimulating factor enhanced migration of bone marrow cells into fibrotic liver and accelerated the regression of liver fibrosis.⁽²⁴⁾ It was also reported that depletion of neutrophils by polymorphonuclear cell-specific antibody treatment blocked degradation of collagen during the recovery process of reversible biliary obstruction in rats.⁽²⁵⁾ In a transgenic mouse (CD11b-DTR) study, depletion of macrophages during the recovery phase after CCl₄-induced injury impaired matrix degradation⁽²⁶⁾; however, because neutrophils also express CD11b, neutrophils could have been depleted in the experimental setting, suggesting that the impaired degradation of matrix could be attributed to neutrophil depletion. Although those reports suggest a positive role for neutrophils in suppressing fibrosis, none provided direct evidence for the antifibrotic role of neutrophils in liver injuries. As described above, the results of our study provide comprehensive evidence showing a beneficial role for neutrophils in liver fibrosis. Furthermore, ectopic expression of *Mmp8* and *Mmp9* suppressed liver fibrosis in wild-type liver (Fig. 6) and immune depletion of neutrophils delayed liver fibrosis resolution (Fig. 7); both of these findings provide a foundation for the antifibrotic

function of neutrophils. Furthermore, neutrophil transfusion into wild-type animals after establishing fibrosis induced rapid resolution of fibrosis (Supporting Fig. S3), suggesting that the antifibrotic function of neutrophils is likely due to fibrosis resolution by expression of *Mmp8* and *Mmp9*.

Infusion of autologous bone marrow cells has been shown to alleviate cirrhosis not only in animal models but also in patients.⁽²⁷⁾ Mesenchymal stem cells (MSCs) and macrophages have been considered the effective cell types in bone marrow cells. In addition to MSCs from bone marrow, MSCs from other tissues, such as adipose tissue, have been shown to be effective.⁽²⁸⁾ However, the mechanism underlying the positive effect of MSCs or macrophages on cirrhosis remains unknown, although several possibilities have been considered; these include transdifferentiation of MSCs into hepatocyte-like cells, secretion of factors that suppress activated stellate cells, modulation of immunity, resolution of fibrosis, and stimulation of hepatocyte proliferation. On the other hand, it has been shown that bone marrow-derived macrophage delivery induced up-regulation of a chemokine that recruits endogenous macrophages and neutrophils in the liver, improving fibrosis.⁽³⁾ Furthermore, hepatic macrophages have been reported to promote the neutrophil-dependent resolution of fibrosis in cholestatic rat liver.⁽²⁹⁾

Taken together with our results, it is strongly suggested that neutrophils may be the cell type that executes the antifibrotic function of bone marrow-derived macrophages. Although the neutrophil has a short lifespan and is rapidly eliminated from the body after infusion, neutrophil infusion quickly ameliorates fibrosis. Thus, if a sufficient number of neutrophils could be prepared, allogenic neutrophils could be a potential source of cell therapy for cirrhosis. Development of neutrophil differentiation from human-induced pluripotent stem cells^(30,31) would be supportive of such a strategy. Furthermore, delivery of chemoattractive chemokines for neutrophils as well as MMP8 and MMP9 in the liver might be an alternative strategy for the treatment of liver fibrosis.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1178/full.